Killing of target cells by redirected granzyme B in the absence of perforin

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Abstract Granzyme B (GzmB) is a potent apoptosis-inducing serine protease of cytotoxic lymphocytes. Following receptor-mediated endocytosis, GzmB is supposed to enter the cytosol through perforin-mediated membrane disruption. We investigated whether retargeting of GzmB to Lewis Y positive surface receptors could lead to perforin-independent target cell death. We coupled recombinant GzmB to the Lewis Y-binding antibody dsFv-B3. Targeting of GzmB to Lewis Y positive cells triggered cell death with similar efficacy as dsFv-B3 targeted *Pseudomonas* exotoxin fragment 38 (PE38). Since GzmB was only weakly inhibited by plasma proteins, GzmB-based immunoconjugates should be useful as a new class of immunotoxins with low immunogenicity utilizing programmed cell death for therapeutic purposes.

Key words: Granzyme B; Apoptosis; Immunotoxin; Lewis Y; Tumor

1. Introduction

As a defence against viral infections and tumor growth, programmed cell death is induced by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells in target cells expressing foreign or altered antigens. One of the activation mechanisms triggering apoptosis involves molecules of secretory granules, in particular the membrane-binding perforin and granzyme B (GzmB), and other members of the family of lymphocyte serine proteases called granzymes. GzmB is one of the most abundant and effective inducers of programmed cell death and activates this program at multiple levels in a caspase-dependent and -independent fashion [1]. The amount of GzmB secreted during target cell recognition into the synaptic cleft is a small fraction of the entire GzmB content of an activated CTL and only a fraction of it presumably reaches the cytosol during target cell attack [2,3]. The natural glycosylated GzmB from cytotoxic granules contains mannose-6-phosphate residues and thus binds to the cation-independent mannose-6-phosphate/insulin-like growth factor receptor (CI-MPR) on target cells [4]. According to the current dogma, CI-MPR-assisted endocytosis and fluid phase accumulation of GzmB in endosomal vesicles are not sufficient to deliver GzmB to the cytosol and to activate the cell death program. In addition to GzmB the co-internalized membrane-bound perforin is required to disturb the trafficking of GzmB-containing vesicles resulting in the delivery of GzmB into the cytosol [5–8].

2. Materials and methods

2.1. Expression and purification of recombinant proteins

The cDNA for human wild-type GzmB was amplified from human bone marrow and cloned into the pET24c(+) *Escherichia coli* expression vector (Novagen). We created the different recombinant GzmB variants using standard polymerase chain reaction (PCR) and cloning techniques. All cloned plasmid sequences were verified by sequencing analyses. Recombinant pro-GzmB carried an N-terminal extension, the propeptide MKH_6 at the N-terminus of the mature GzmB starting with Ile-Ile-Gly-Gly. This precursor protein was expressed, purified and then converted to active GzmB as described for granzyme K [9]. The concentration of all proteins was determined spectroscopically using the appropriate extinction coefficient at 280 nm. The viral serpin *cytokine response modifier A* (*CrmA*) was expressed as described [10]. The cDNA plasmid in the pFLAG-1 vector was a generous gift of G. Salvesen. The disulfide-linked B3-R_8C (dsFv-B3-R_8C), an engineered Fv fragment of the B3 antibody with the C-terminal R_8CP extension, was expressed and refolded as described [11,12]. The two components B3-R_8C and GzmB-CD_8 were incubated for 2 h at room temperature (RT) in 25 mM Na_2HPO_4, 25 mM KH_2PO_4 (pH 8.0), 2 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM reduced glutathione (GSH). The pH was then shifted to 4.5, and both proteins were dialyzed at 4°C against 25 mM Na_2HPO_4, 25 mM KH_2PO_4 (pH 4.5), 2 mM EDTA. The free cysteine of B3-R_8C was activated at pH 8.0 and RT for 1 h by adding DTP (2,2′-dithiopyridine; Sigma) at a 10-fold molar excess. Free DTP was removed by gel filtration (PD-10; Pharmacia). The activated B3-R_8C was incubated with GzmB-CD_8 at a molar ratio of 3:1 at 20°C in 25 mM Na_2HPO_4, 25 mM KH_2PO_4 (pH 8.1), 2 mM EDTA for 4 h. The resulting conjugate was separated from the uncoupled components by cation exchange chromatography (Poros HS) using 50 mM Tris (pH 7.4), 200 mM NaCl, 1 mM EDTA and a linear NaCl gradient from 0.2 to 2 M.

2.2. Activity of GzmB in plasma

GzmB was preincubated at RT with 5% human plasma. After 30 min $CASP7^{C285A}$, a gift from G. Salvesen, was added [13]. Cleavage of $CASP7^{C285A}$ was analyzed under reducing conditions by Coomassie-stained 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Enzymatic activity of 200 nM GzmB was measured using the substrate Ac-IEPD-pNA (Calbiochem) at a final concentration of 250 μM in 100 mM Tris pH 8.1, 100 mM NaCl, 0.05% Triton X-100. Absorption of the resulting cleavage product was measured at

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Abbreviations: α_1-AT, α_1-antitrypsin; BSA, bovine serum albumin; CI-MPR, cation-independent mannose-6-phosphate/insulin-like growth factor receptor; CrmA, cytokine response modifier A; CTL, cytotoxic T lymphocytes; FACS, fluorescence-activated cell sorter; GzmB, granzyme B; NK, natural killer; PBS, phosphate-buffered saline; PE38, *Pseudomonas* exotoxin fragment 38; PI, propidium iodide; PR3, proteinase 3; RT, room temperature; SLO, streptolysin O

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405 nm using a Dynatech MR4000 plate reader. Incubations of 1 μM GzmB were performed with 3 μM CrmA at 37°C for 30 min as described [10]. Incubations of GzmB and proteinase 3 (PR3) in human EDTA plasma or α_1-antitrypsin (α_1-AT) (Sigma) were performed at 37°C for 20 min. Remaining enzymatic activities were measured thereafter. PR3 activity (200 nM) was determined by hydrolysis of the tetrapeptide substrate MeOSuc-AAPV-pNA (1 mM; Bachem). Background absorption of human plasma was subtracted.

2.3. Cell culture

K562 cells were grown in RPMI 1640, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 0.1 mM 2-mercaptoethanol. SK-BR3 cells were grown in Dulbecco's modified Eagle's medium (DMEM), 1% GlutaMAX I, 4.5 g/l D-glucose and Na-pyruvate, 10% FBS and 0.5% gentamicin. MCF7, MCF7^{casp3} and A431 cells were cultured in RPMI 1640 Dutch modification (Gibco Lifescience), 10% FBS, 1% GlutaMAX, and 0.5% gentamicin.

2.4. Short-term apoptosis induction

Adherent cells were trypsinized and resuspended in FBS-containing medium. Cells were washed three times in serum-free medium containing 0.5% bovine serum albumin (BSA) (Sigma). Apoptosis experiments were performed in 100 μl with 10^5 cells per well in serum-free 0.5% BSA medium. GzmB was added to the cells before the addition of streptolysin O (SLO). SLO (25 μg/ml) was activated in medium containing 40 μM cysteine for 10 min at RT, diluted and freshly added to the cells at a final concentration of 150 ng/ml. After 4–5 h incubation adherent cells were trypsinized, washed in fluorescence-activated cell sorter (FACS) medium (phosphate-buffered saline (PBS), 3% FBS, 0.01% NaN_3) and subsequently in PBS. Cells in a volume of 25 μl were stained for 15 min at RT with fluorescein isothiocyanate (FITC)-annexin V (Caltech) and propidium iodide (PI) 1:20 (BD-Biosciences) in binding buffer (BD-Biosciences), diluted and analyzed by FACS.

2.5. Detection of Lewis Y expression

B3-R_8C was labeled with TexasRed™ (Molecular Probes) via the free cysteine. Cells were trypsinized, washed and resuspended in FACS medium. Cells (10^5) were resuspended in 25 μl of FACS medium and stained with the indicated antibody dilution at 4°C for 60 min. In parallel a live gate was defined by PI staining. Then cells were washed and analyzed by FACS.

2.6. Cytotoxic action of immunotoxins

Different cell lines were incubated with the immunoconjugates B3-GzmB, B3-*Pseudomonas* exotoxin fragment 38 (PE38) or the respective unconjugated enzymes, GzmB-CD_8 and E_8C-PE38 [11]. Since K562 and A431 were Lewis Y negative, the Lewis Y positive SK-BR3, MCF7 and MCF7^{casp3} cells were used as targets. The cells were cultured until they reached 20% confluency. The immunotoxins or control proteins were prediluted in PBS and added to the cells in medium containing 10% FBS. After 48 h cells were harvested by trypsinization, washed, resuspended in 10 mM HEPES/NaOH, 140 mM NaCl, 5 mM $CaCl_2$ (pH 7.4) and stained with PI. The percentage of viable cells was determined by FACS analysis. The data were fitted according to the equation: $Y = a + b/(1+10^{(\log(x)-\log(EC_{50}))})$, where a = % surviving cells at the highest toxin concentration; b = % of surviving cells at the lowest toxin concentration; x = toxin concentration.

3. Results and discussion

Following procedures previously described [9,14], we generated large amounts of non-glycosylated wild-type GzmB and a catalytically inactive variant. This recombinant GzmB does not bind to the ubiquitously expressed CI-MPR and can enter target cells by fluid phase uptake [15]. Firstly, we tested its apoptosis-inducing potential in cell culture, using K562 cells as targets and sublytic concentrations of SLO as a perforin substitute. After 5 h of incubation with SLO and GzmB, we observed annexin V binding to the cellular membrane in 80% of all K562 cells indicating apoptotic cell death (Fig. 1A).

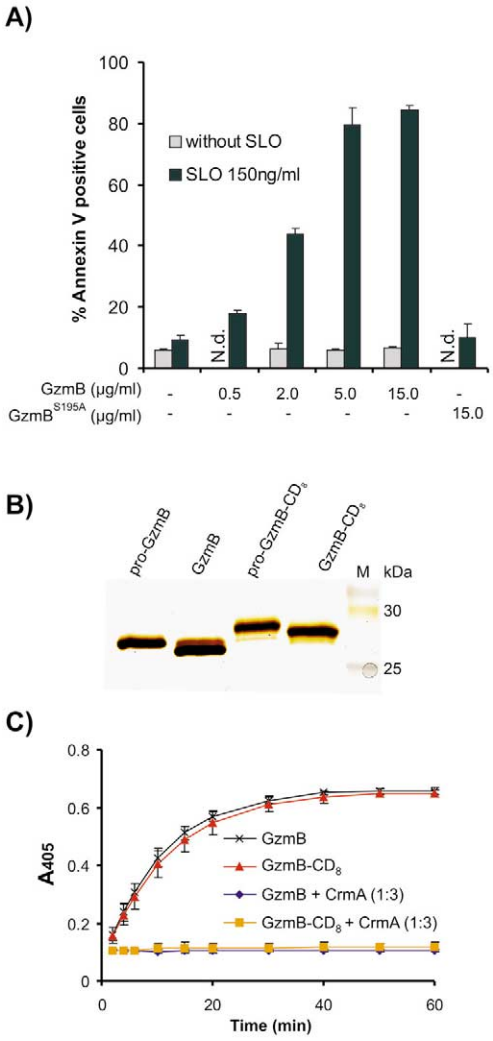

Fig. 1. Preparation of GzmB and GzmB-CD_8. A: Apoptosis induction by recombinant GzmB. K562 cells were treated with increasing concentrations of recombinant GzmB and subsequently exposed to sublytic amounts of SLO (150 ng/ml) for 5 h. Incubation with the active site mutant GzmB^{S195A} in the presence of SLO and incubations with GzmB in the absence of SLO (gray bars) served as negative controls. FITC-annexin V binding records both early apoptotic (PI negative) and late apoptotic cells, which are PI positive. The diagrams (A, C) represent mean values of triplicate measurements with standard deviations (N.d.: not determined). B: The refolded proteins MKH_6-GzmB and MKH_6-GzmB-CD_8 (0.5 μg per lane) before and after N-terminal trimming by bovine cathepsin C, were analyzed by silver-stained SDS–PAGE. The shift indicates the propeptide (MKH_6) removal. C: Catalytic activity of recombinant GzmB and GzmB-CD_8. Both enzyme preparations show amidolytic activities towards a tetrapeptide substrate and are inhibited by recombinant CrmA, which was added at a molar ratio of 1:3 in excess of GzmB. Hydrolysis of the substrate Ac-IEPD-pNA was determined by absorption (A) at 405 nm.

Likewise, Jurkat cells were killed with similar efficiency (data not shown) by GzmB in the presence of SLO, but not in its absence. A catalytically inactive GzmB variant (Ser195-Ala) did not induce apoptosis excluding unspecific cytotoxic effects of the recombinant protein (Fig. 1A). These data clearly demonstrate that non-glycosylated GzmB is relatively non-toxic to eukaryotic cells as long as it is not delivered to the cytosol by membrane-disrupting agents.

To achieve a highly efficient endocytic uptake of recombi-

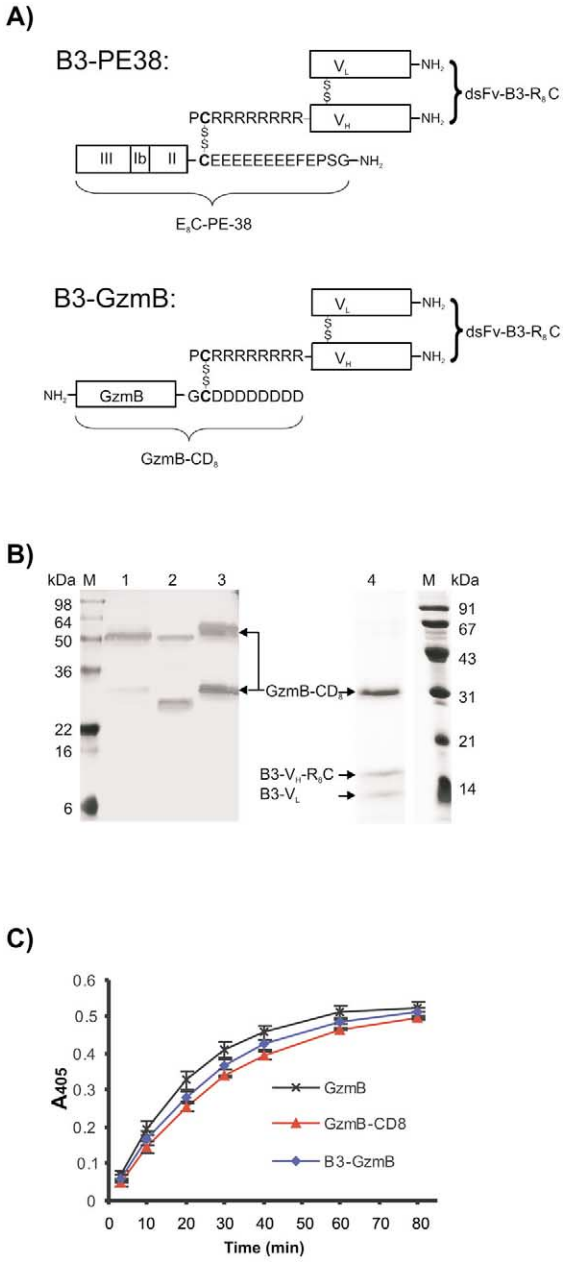

Fig. 2. Generation and characterization of immunoconjugates. A: Schematic representation of B3-PE38 and B3-GzmB. Polyionic adapter peptides containing a cysteine residue were genetically fused to the heavy chain C-terminus of the disulfide-linked dsFv-B3 antibody, to the C-terminus of GzmB and to the N-terminus of PE38. Heterodimeric B3-GzmB conjugates that form via charge–charge interactions are stabilized by an intermolecular disulfide bond. Amino-termini are indicated by 'NH_2'. B: SDS–PAGE analysis of the B3-GzmB conjugate and single components under non-reducing conditions: lane 1, B3-GzmB, 53.8 kDa; lane 2, dsFv-B3-R_8C; lane 3, GzmB-CD_8. Due to the free C-terminal cysteine residue, both GzmB-CD_8 and B3-R_8C exist as monomers and homodimers (upper band in lanes 2 and 3) after refolding. In addition we analyzed B3-GzmB under reducing conditions (lane 4). Protein bands were silver (lanes 1–3) or Coomassie blue stained (lane 4). C: Enzymatic activity of B3-GzmB in comparison to GzmB and GzmB-CD_8. The enzymes (100 nM) show similar catalytic activity towards the tetrapeptide Ac-IEPD-pNA substrate. Hydrolysis of the substrate was determined by absorption (A) at 405 nm. Background absorption was subtracted.

nant non-glycosylated GzmB by membrane targeted delivery, we selected the disulfide stabilized Fv fragment (dsFv) of the monoclonal antibody B3 recognizing the Lewis Y carbohydrate antigen [16]. This antigen is highly expressed on the surface of many breast, colon, lung and epidermal tumor cells [17] and is endocytosed continuously. B3 antibodies conjugated to the PE38 have been approved for a number of clinical trials [18–20]. To ensure a stoichiometric coupling between GzmB and the dsFv targeting antibody, which carried a positively charged peptide (R_8CP) at the C-terminus of the

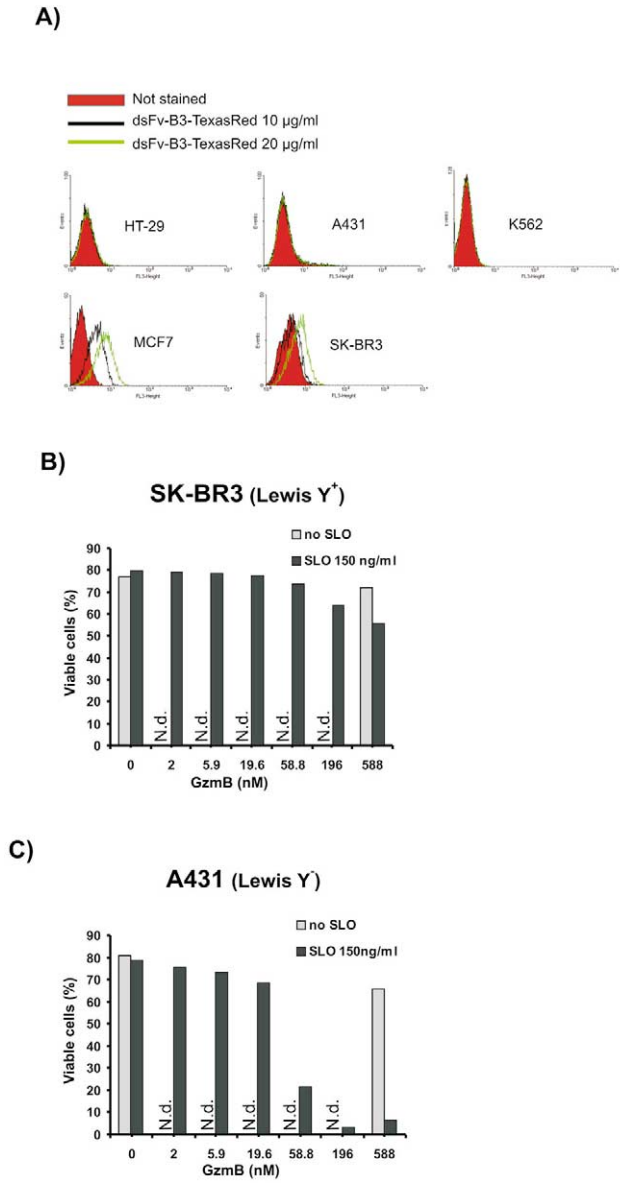

Fig. 3. Characterization of different cell lines. A: FACS analyses of cell lines used in this paper. Cells were stained with TexasRed™-labeled dsFv-B3. HT-29 cells are shown as negative controls expressing no Lewis Y. Our A431 line as well as K562 cells are clearly negative for dsFv-B3 binding whereas MCF7 and SK-BR3 cells are positive. B,C: Killing of Lewis Y positive SK-BR3 and Lewis Y negative A431 cells by GzmB in the presence of sublytic amounts of SLO. Apoptosis induction by GzmB in the presence of SLO was recorded by FACS analysis using FITC-annexin V and PI staining. Black bars indicate the percentage of viable non-apoptotic cells after 4.5 h of SLO and GzmB exposure, which are both annexin V and PI negative (N.d.: not determined).

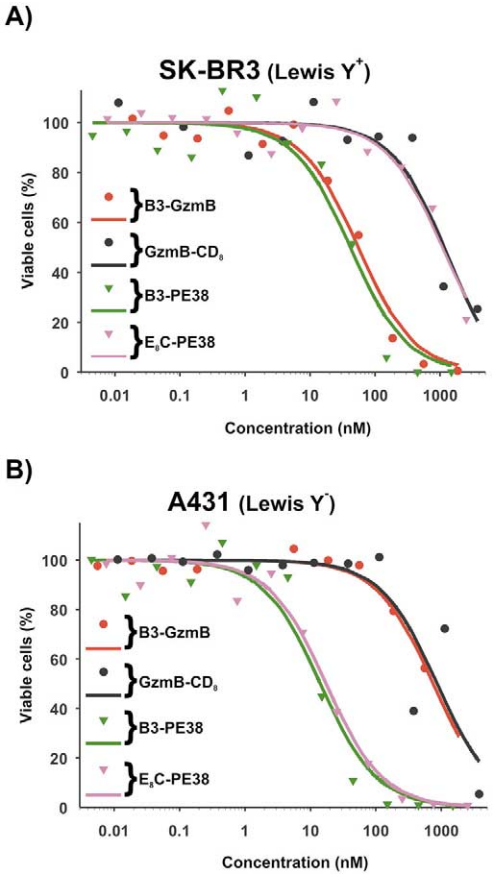

Fig. 4. Biological activity of antibody targeted GzmB. Cytotoxicity against A: Lewis Y positive SK-BR3 and B: Lewis Y negative A431 cells by B3-GzmB in comparison to free GzmB-CD_8 and targeted B3-PE38 and free E_8C-PE38. Cytotoxicity of immunotoxins and free toxins after 48 h incubation was analyzed by PI staining, which encompasses late-stage apoptotic and necrotic cells. The number of unstained (viable) cells was determined by FACS analysis. Shown is the percentage of viable cells in relation to the average of viable cells treated with minimal toxin concentrations. Cues represent single data points, lines show cytotoxic fit. The EC_{50} values (half-maximal effect concentration) are given in Table 1.

V_H chain [11,12], we fused a negatively charged peptide sequence, GCD_8, to the C-terminus of the natural GzmB sequence (GzmB-CD_8, Fig. 1B). Inclusion bodies obtained for GzmB-CD_8 were solubilized, refolded and finally converted to active GzmB with the help of cathepsin C (Fig. 1B). Fig. 1C shows that both forms, GzmB and GzmB-CD_8, have similar activities towards the caspase 3-derived peptide substrate Ac-IEPD-pNA and are inhibited equally well by the cowpox serpin CrmA, indicating that both preparations were correctly refolded.

The GzmB-CD_8 variant was then coupled to the recombinant disulfide stabilized Fv fragment of B3 via its positively charged R_8CP peptide at the C-terminus of the V_H chain [11,12] (Fig. 2A). In this way, a high yield of the B3-GzmB immunoconjugate with the expected size of 53.8 kDa was obtained (Fig. 2B, lane 1). The conjugate split into its three polypeptide chains upon reduction (Fig. 2B, lane 4). Both GzmB-CD_8 and B3-GzmB showed comparable enzymatic activities (Fig. 2C).

To identify suitable sensitive target cells for redirected GzmB, we analyzed Lewis Y expression and apoptosis induction by GzmB in the presence of sublytic SLO concentrations. The A431 cell line derived from a human vulvar squamous carcinoma, was previously used as a Lewis Y antigen-expressing target [17], but the sublines we obtained turned out to be Lewis Y negative in FACS analyses (Fig. 3A). SK-BR3, a human breast carcinoma cell line, however, was clearly positive. To determine the relative sensitivity of these two cell lines towards apoptosis induction by GzmB, we exposed SK-BR3 and A431 cells to sublytic amounts of SLO and increasing GzmB concentrations in short-term experiments (Fig. 3B and C). We observed significant differences in SLO-facilitated target cell killing. A431 cells were highly sensitive to GzmB-induced apoptosis, whereas SK-BR3 cells appeared to be relatively resistant.

When we incubated the Lewis Y positive cell line SK-BR3 with the B3-GzmB immunoconjugate in the absence of SLO for 48 h, we observed specific killing of these target cells (Fig. 4A), whereas B3-GzmB displayed no net effect on Lewis Y negative A431 cells by comparison with untargeted GzmB-CD_8 (Fig. 4B, Table 1). As further Lewis Y positive cell lines, we used MCF7 cells naturally deficient for caspase 3 and MCF7 cells stably transfected with caspase 3, $MCF7^{casp3}$ [21]. The caspase 3 positive variant was also specifically killed by B3-GzmB conjugates, in contrast to the caspase 3 negative wild-type cells (Table 1) suggesting that the proapoptotic action of GzmB was amplified by caspase 3 in $MCF7^{casp3}$ cells. Our experiments clearly show that GzmB retargeted to Lewis Y positive surface structures can induce cell death without endosome-disrupting agent. These conjugate-specific effects occurred at more than 10-fold lower concentrations than required to generate non-targeted cellular toxicity.

In view of the fact that the Lewis Y-specific B3 antibody binds to many different carbohydrate-containing proteins on the surface of tumors and epithelial cells [17], it is difficult to envisage how GzmB becomes translocated across the plasma membrane. After binding of dsFv-B3 to the surface, the complex is internalized via Lewis Y positive receptors and accumulates in endocytic transport vesicles. Due to the lower endosomal pH, GzmB conjugates may dissociate from the surface antigen and leak into the cytosol inside the cell. Some of the endocytosed receptor–immunoconjugate complexes may well be translocated into the cytosol [22] and activate proapoptotic substrates before reaching the proteasomes for degradation. Our observation that B3-GzmB conjugates

Table 1
Half-maximal effect concentrations for immunotoxins and free toxins

Cell lines	Lewis Y	EC_{50} (nM)[a]			
		E_8C-PE38	B3-PE38	GzmB-CD_8	B3-GzmB
A431	Negative	17	14	863	751
K562		37	14	>1128[b]	>557[b]
MCF7	Positive	137[c]	1.7[c]	198	140
$MCF7^{casp3}$		58	1.8	394[d]	35
SK-BR3		1100	42	1595[d]	98[e]

[a] EC_{50} indicates the concentration of immunotoxin or free toxin that shows 50% cytotoxicity towards the cell lines given.
[b] EC_{50} not determined, out of range tested.
[c] Determined by Kleinschmidt et al. using the same method as published [11].
[d] Mean of data obtained from two independent experiments.
[e] Mean of data obtained from three independent experiments.

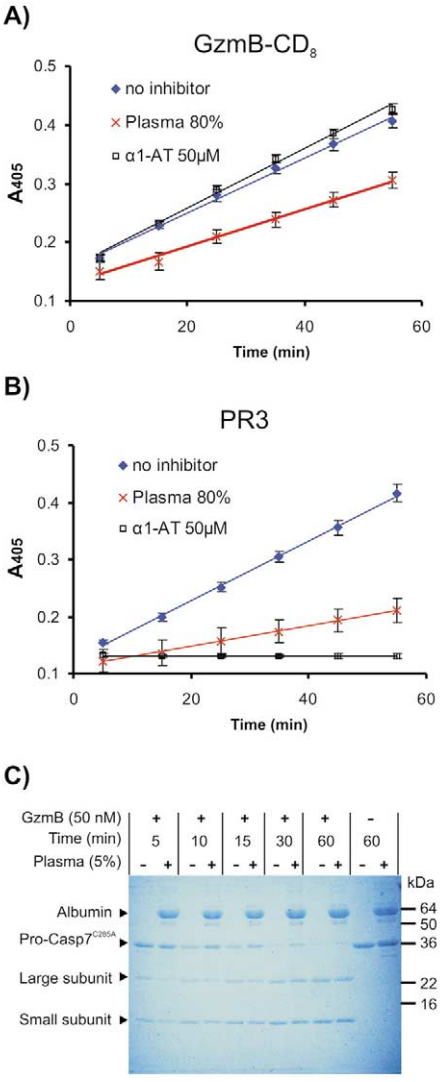

Fig. 5. Activity of GzmB in plasma. A: GzmB is not inactivated by plasma proteins and purified α_1-AT. 1 μM of GzmB-CD_8 was pre-incubated with 50 μM α_1-AT or 80% human EDTA plasma for 20 min at 37°C. Shown is the increase of light absorbance at 405 nm over time indicating the hydrolysis of the substrate Ac-IEPD-pNA. B: Inhibition of 1 μM PR3 by EDTA plasma and purified α_1-AT was recorded by measuring the hydrolysis of the PR3 substrate MeOSuc-AAPV-pNA. As is known, PR3 is inactivated by α_1-AT and α_2-macroglobulin, but the PR3–α_2-macroglobulin complex is still active on small peptide substrates. C: Recombinant GzmB cleaves pro-$CASP7^{C285A}$, a catalytically inactive mutant pro-caspase 7, in the presence of plasma proteins. SDS–PAGE analyses of 5% plasma spiked with 50 μM pro-$CASP7^{C285A}$ and 50 nM GzmB. The catalytically inactive C285A mutant of caspase 7 was used to exclude autocatalytic activation.

and GzmB alone did not display toxicity toward Lewis Y negative K562 and A431 over a broad range of concentrations is consistent with a cytosolic proapoptotic function of targeted GzmB after translocation.

Since it is known that the PE38 fragment in contrast to GzmB utilizes a cellular mechanism for retrograde entry into the cytosol [23], we compared the high efficacy of targeted PE38 with that of targeted GzmB under identical conditions. Indeed, B3-PE38 conjugates were effective at 20- and 2-fold lower concentrations than targeted GzmB towards $MCF7^{casp3}$ cells and SK-BR3 cells, but unspecific toxicity of B3-PE38 against Lewis Y negative cells appeared already at 14 nM (Table 1). By contrast, extracellular GzmB-CD_8 showed significantly lower toxicity (EC_{50} values of 863 nM) than free E_8C-PE38 towards A431 cells (Table 1) despite their high sensitivity to cytosolic GzmB (Fig. 3C). Conversely, SK-BR3, a relatively resistant target cell line (Fig. 3B), was killed with similar efficacy by B3-GzmB in comparison with the B3-PE38 conjugate. Half-maximal effective concentrations for both immunoconjugates were at least one order of magnitude lower than the respective EC_{50} values of the free toxins. We, therefore, conclude that dsFv-B3-mediated targeting to Lewis Y positive cell surface receptors improved the cytosolic uptake and biological efficacy of GzmB similarly to that of PE38.

In view of these surprising findings we considered GzmB as a new surrogate for heterologous toxin components. As one major concern is the inactivation of human GzmB by extracellular proteinase inhibitors, we investigated its inhibition by human plasma and α_1-AT, a major plasma serine protease inhibitor of the serpin class. Excess of purified α_1-AT completely inactivated the neutrophil-derived PR3, but did not inhibit recombinant GzmB-CD_8 (Fig. 5A,B) contradicting previous findings [24]. The incubation of GzmB-CD_8 in 80% plasma for 20 min at 37°C lead to a moderate decrease of activity of about 30% (Fig. 5A), whereas PR3 was inhibited by about 70% (Fig. 5B). To exclude the formation of GzmB–α_2-macroglobulin complexes, which still hydrolyze small substrates, but no longer cleave macromolecular substrates, we analyzed the pro-caspase 7-activating property of recombinant GzmB in the presence and absence of 5% human plasma. The addition of plasma to recombinant GzmB delayed cleavage of the active site mutant pro-caspase 7 insignificantly (Fig. 5C).

Very recently when this work was reviewed, two reports have been published in which GzmB was similarly used as an effector domain and fused to targeting sequences [25,26]. GzmB covalently linked to VEGF sequences [25] and to an antibody fragment (scFvMEL) against a melanoma antigen [26] was shown to be taken up into the cytosol and was cytotoxic towards receptor or antigen-carrying target cells. Human GzmB is a highly specialized serine protease of lysosome-like granules with unusual caspase-activating properties and intrinsic stability in extracellular body fluids and lysosome-like granules of T and NK cells. Since humans are supposed to have a strong immunological tolerance against endogenous GzmB, a humoral or cellular immune response against GzmB should not occur. In this regard, GzmB is superior to most other toxins, e.g. *Diphtheria* toxin, the plant-derived ricin toxin A chain or the *Pseudomonas* exotoxin which are highly immunogenic [18]. Coupling to other antibodies or receptor ligands and specific retargeting to receptors that enhance its endocytic uptake and accumulation should broaden the applicability and safety of GzmB-based immunoconjugates beyond the level of present-day immunotoxins.

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